

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of: **Waldman, Scott A.**

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Title: **METASTATIC COLORECTAL CANCER VACCINE**

Commissioner for Patents  
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Dear Sir:

**DECLARATION OF DR. SCOTT A. WALDMAN**

I, Scott A. Waldman, do hereby declare as follows:

1. I am the inventor of the subject matter claimed in the above-identified U.S. Patent Application.
2. I am a Professor at the Thomas Jefferson University, Jefferson Medical College where I have appointments in both the Department of Medicine, Division of Clinical Pharmacology, and the Department of Biochemistry & Molecular Pharmacology.

3. I am co-author of the following published scientific articles:

a. Snook, A.E. *et al.* Cancer mucosa antigens as a novel immunotherapeutic class of tumor-associated antigen (2007) Clin Pharmacol Ther. 82(6):734-739, Review., a copy of which is attached hereto as Exhibit A.

b. Snook, A.E. *et al.* Guanylyl cyclase C -induced immunotherapeutic responses opposing tumor metastases without autoimmunity (2008) *Submitted*, a copy of which is attached hereto as Exhibit B.

4. Snook, A.E. *et al.* Cancer mucosa antigens as a novel immunotherapeutic class of tumor-associated antigen (2007) Clin Pharmacol Ther. 82(6):734-739, reports the potential of antigens whose expression is restricted to normal intestinal mucosa and derivative colorectal tumors as a class of immune targets supporting efficacious anti-tumor immunotherapy. The article notes that GCC, whose expression is restricted to normal intestine and derivative tumors, is an ideal candidate for clinical study and includes data in Figure 3 showing results of experiments of GCC-specific anti-tumor immunity in a mouse model. The article reports that the results provide proof of principle.

5. Snook, A.E. *et al.* Guanylyl cyclase C -induced immunotherapeutic responses opposing tumor metastases without autoimmunity (2008) *Submitted*, reports the antitumor efficacy of guanylyl cyclase C (GCC) delivered using a recombinant viral vector to mouse models of metastatic colon cancer. The manuscript reports that immunization with GCC-expressing viral vectors opposed the formation of nascent metastases to liver and lung, and extended the median survival of mice with established lung metastases following therapeutic immunization without autoimmunity.

6. I declare that all statements made herein are of our own knowledge true and statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

\_\_\_\_\_  
Scott A. Waldman, M.D., Ph.D.

\_\_\_\_\_  
Date

Attachments:

Exhibit A - Snook, A.E. *et al.* Cancer mucosa antigens as a novel immunotherapeutic class of tumor-associated antigen (2007) Clin Pharmacol Ther. 82(6):734-739

Exhibit B. - Snook, A.E. *et al.* Guanylyl cyclase C -induced immunotherapeutic responses opposing tumor metastases without autoimmunity (2008) Submitted

# EXHIBIT A

# Cancer Mucosa Antigens as a Novel Immunotherapeutic Class of Tumor-associated Antigen

AE Snook<sup>1</sup>, LC Eisenlohr<sup>2</sup>, JL Rothstein<sup>3</sup> and SA Waldman<sup>1</sup>

Colorectal cancer is a leading cause of cancer-related mortality worldwide. Surgery and chemoradiation exhibit incomplete efficacy and, ultimately, 50% of patients die of metastatic disease. In the context of that unmet clinical need, immunotherapeutic approaches have enjoyed limited success, partly because of a paucity of suitable antigen targets. However, exploitation of immune compartmentalization, employing antigens with expression restricted to normal intestinal mucosa and derivative colorectal tumors—cancer mucosa antigens (CMAs)—may represent a previously unrecognized class of immune targets supporting efficacious antitumor immunotherapy. Guanylyl cyclase C (GCC) is an intestine/colorectal cancer-restricted protein ideally suited as the first CMA for clinical evaluation.

## COLORECTAL CANCER

Colorectal cancer is the third leading cause of cancer-related deaths in the United States, producing ~500,000 deaths per year worldwide.<sup>1</sup> Mortality reflects the presence of metastases at the time of diagnosis in ~20% of patients and their development during the course of disease in ~35% of patients. Current clinical practice employs surgery and, for advanced disease, adjuvant fluoropyrimidine therapy. Nevertheless, in patients treated with the presumption of cure, disease recurrence, with metastases primarily to lung and liver, produces an overall 5-year mortality of ~35%.

This morbidity and mortality underscores the unmet therapeutic need in colorectal cancer. In that context, there is an established relationship between immune infiltration and prognosis in colorectal cancer patients,<sup>2</sup> suggesting that spontaneous anticolorectal cancer immune responses favorably impact clinical outcome. The type, density, and location of infiltrating immune cells within the tumor are critical for prognostic significance, and patients with CD3<sup>+</sup> and

CD45RO<sup>+</sup> cells in the tumor center exhibit a significant survival advantage.<sup>3</sup> These immune parameters were significantly better prognostic indicators than current histopathological methods.<sup>3</sup> Antigens targeted by infiltrating lymphocytes include Ep-CAM, her-2/neu, carcinoembryonic antigen, and squamous cell carcinoma antigen recognized by T cells (SART-3), and mutated proteins such as transforming growth factor  $\beta$  receptor II (TGF $\beta$ /RII, Cdx2, and K-ras antigens.<sup>4</sup>

These observations suggest that immunological targeting may offer a therapeutically advantageous approach to the management of patients with colorectal cancer. Clinical studies of immunotherapy in colorectal cancer have focused on three classes of tumor antigens: mutated oncogenes, oncofetal/cancer testis (CT) antigens, and overexpressed self-antigens.<sup>4</sup> Although results targeting mutated oncogenes such as p53 and K-ras have been encouraging,<sup>5–7</sup> immunotherapy directed against patient-specific mutations is impractical on a global scale, and the field has focused on oncofetal/CT antigens and overexpressed self-antigens shared among patients.<sup>8</sup> The overexpressed self-antigen, carcinoembryonic antigen, has been the most extensively studied, targeted in ~50% of clinical trials.<sup>8</sup> Unfortunately, active specific immunotherapy for colorectal cancer has had remarkably poor efficacy, resulting in a clinical response rate of <1% from >30 studies examining >500 patients.<sup>8</sup> Comparison with immunotherapy to melanoma, which has enjoyed the greatest relative success, suggests a glaring absence of differentiation antigens targeted in colorectal cancer.<sup>4</sup> Their omission may reflect consideration of collateral autoimmune disease or a presumed lack of efficacy in targeting self-antigens. However, the mucosal-specific expression of differentiation antigens, in the context of compartmentalization of mucosal and central immune responses, suggests that these antigens may offer a unique immunotherapeutic opportunity in colorectal cancer.

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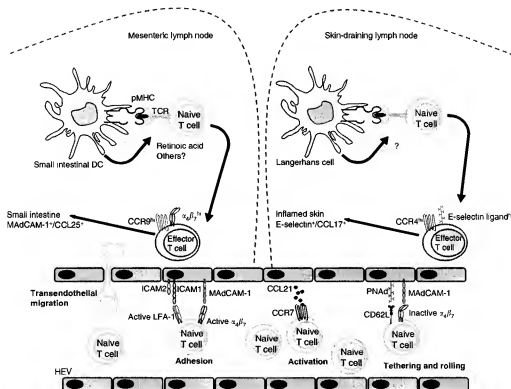
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## IMMUNE COMPARTMENTALIZATION

The central compartment is a sterile environment composed of self-antigens, and the immune system therein defends against invading microorganisms. Conversely, the mucosal compartment forms a barrier to the outside environment and is composed of commensal and pathogenic microorganisms, food and self-antigens, and environmental toxins. The mucosal immune system maintains a complex balance between unresponsiveness to food and self-antigens and defensive immunity to commensal and pathogenic organisms. Moreover, structural and functional compartmentalization is reflected by the tissue-restricted recirculation characteristics of lymphocytes from the two compartments such that central compartment immune responses rarely extend to mucosal surfaces.<sup>9–11</sup> The local microenvironment, particularly resident antigen-presenting cells, imprints T cells upon activation with circulation characteristics such that

homing to lymphoid tissues is downregulated, whereas that to associated tissues is upregulated (Figure 1). Immune compartmentalization contributes significantly to immunity against invading microorganisms and prevents autoimmune disease by increasing the efficiency of regional immune responses and decreasing tissue antigen cross-reactivity.<sup>12</sup>

Independence of immune responses in mucosae is reminiscent of immunologically privileged tissues, such as the eye, testis, ovary, brain, and pregnant uterus, wherein the central immune responses rarely extend to privileged compartments. This immunological independence has been exploited to generate therapeutic responses to cancers employing antigens that are normally restricted to testis but ectopically expressed during carcinogenesis—CT antigens.<sup>13</sup> The advantages of these antigens include the potential limited central compartment tolerance opposing antitumor immunity and the absence of compartment crosstalk limiting



**Figure 1** Tissue-specific tropism mediated by T cell imprinting. Naive T cells expressing the adhesion molecule CD62L and the CC-chemokine receptor 7 (CCR7) circulate through the blood and lymph and enter into secondary lymph organs where antigen-specific activation occurs. This well-characterized process requires lymphocyte tethering to, and rolling on, high endothelial venules (HEVs) mediated by CD62L binding to peripheral node addressin (PNA) on HEVs—Peyer's patch HEVs lack PNA expression, resulting in the use of a secondary binding mechanism through interactions of mucosal addressin cell-adhesion molecule 1 (MAdCAM-1) with  $\alpha_4\beta_7$  integrin. CC-chemokine ligand 21 (CCL21)-dependent activation of lymphocyte function-associated antigen 1 (LFA-1) or  $\alpha_4\beta_7$  integrin results in adhesion to the endothelium, followed by transendothelial migration into secondary lymphoid organs such as intestine- or skin-draining lymph nodes. There, mature dendritic cells, having acquired antigens in tissues, activate antigen-specific T cells through specific T cell receptor (TCR) interactions with peptide-MHC (pMHC) complexes. Dendritic cells activated in particular tissues also "imprint" T cells with new homing characteristics such that migration to lymphoid organs is downregulated, whereas new receptor combination expression mediates selective migration to tissues from which dendritic cells acquired antigens. Upon mucosal antigen exposure, small intestinal dendritic cells activate naive T cells and, in a retinoic acid-dependent manner, induce upregulation of  $\alpha_4\beta_7$  integrin and CCR9, resulting in selective migration to MAdCAM-1/CCL25-expressing small intestine. On the other hand, dendritic cells from central compartment tissues such as skin imprint T cells with different homing characteristics, reflecting alternative surface receptor utilization. T cells imprinted by epidermal dendritic cells (Langerhans cells) upregulate CCR4 and E-selectin ligand expression, mediating homing to inflamed skin, where the endothelium coordinately upregulates E-selectin and CCL17 expression. DC, dendritic cell; ICAM1/2, intercellular adhesion molecule 1/2.

autoimmune disease. However, with the exception of melanoma and lung cancers, these antigens are suboptimal targets, as individual CT antigens are not universally associated with disease, with expression in <20% of epithelial cancers, including colorectal tumors.<sup>14</sup> In that context, the universal expression of mucosal self-antigens by tumors derived therein may provide a solution for targeted immunotherapy. Like CT antigens, central compartment tolerance to mucosal self-antigens may be limited, allowing the generation of efficacious antitumor immunity, although such antigens should not elicit mucosal autoimmune disease if employed for vaccination in the central compartment against metastatic tumors (Figure 2). Employing mucosal self-antigens expressed by normal intestinal epithelium and derivative tumors for targeted immunotherapy of colorectal cancer would represent a significant paradigm shift, as such antigens have not been explored previously. We have recently proposed these antigens as a novel class of tumor-associated antigen, CMAs,<sup>4</sup> and have explored the immunogenicity and antitumor efficacy of a model CMA, GCC, in animal models (A. Snook *et al.*, unpublished data).

#### GUANYLYL CYCLASE C

GCC is the receptor for the endogenous hormones guanylin and uroguanylin and for the diarrheagenic bacterial heat-stable enterotoxins.<sup>15</sup> As a member of the guanylyl cyclase family of receptors, GCC converts cytosolic GTP to the second

messenger cyclic guanosine monophosphate (GMP), resulting in activation of numerous downstream signaling pathways.<sup>15</sup> GCC is expressed selectively by intestinal epithelial cells from the duodenum to the rectum, where it regulates physiological processes, including enterocyte proliferation, migration, differentiation, and metabolism.<sup>16</sup> Importantly, expression persists through all stages of colorectal carcinogenesis, including primary and metastatic tumors,<sup>17–20</sup> suggesting potential utility for GCC-targeted therapeutics for colorectal cancer.<sup>16</sup>

#### Universal expression of GCC by gastrointestinal malignancies

GCC mRNA was detected in >100 patient samples, including normal intestine, primary colorectal tumors, and colorectal cancers metastatic to various tissues, but not in >200 samples from extraintestinal tissues.<sup>4</sup> Moreover, GCC protein expression, quantified by ligand binding or immunohistochemistry, was identified in 100% of intestinal specimens, including normal intestine and primary and metastatic colorectal tumors (>100 specimens) but not in normal extraintestinal tissues or tumors (>100 specimens). GCC is also overexpressed in premalignant intestinal conditions, including hyperplastic polyps, tubular adenomas, and inflammatory bowel disease.<sup>17</sup> Furthermore, although GCC expression is absent from normal or inflamed tissues of the upper gastrointestinal (GI) tract, it is expressed in all cases of dysplasia and adenocarcinomas arising from intestinal metaplasia in the esophagus and stomach,<sup>17</sup>



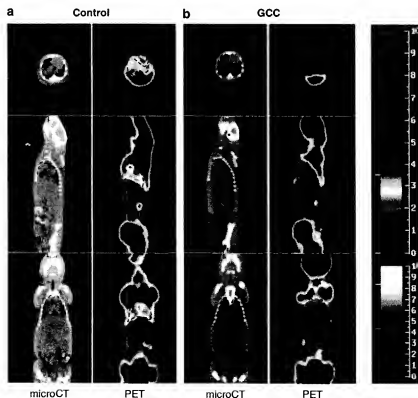
**Figure 2** Cancer mucosa antigen (CMA)-targeted immunotherapy. Mucosal self-antigens comprise a novel class of tumor-associated antigens termed CMAs. (1) CMA immunization regimens delivered to the central immune compartment (such as a subcutaneous injection) are acquired by professional antigen-presenting cells (APCs), which process the antigen into small fragments and present them on their surface for immune system surveillance. Adjuvants within the immunization also provide “danger signals” to APCs, resulting in their maturation and acquisition of receptor expression necessary for T cell activation. (2) Mature, antigen-loaded APCs then travel via the lymphatics to the draining lymph nodes and to the spleen. (3) There, CMA-specific T cells are activated by APCs, acquiring effector functions such as cytotoxicity, and are imprinted with homing characteristics for central compartment tissues (see Figure 1). (4) Activated CMA-specific T cells travel to central compartment tissues such as liver containing CMA-expressing colorectal cancer (CRC) metastases. There, T cells exert their antitumor function by directly killing tumor cells or by altering the tumor microenvironment. (5) However, activated CMA-specific T cells are excluded from entering mucosal tissues, including intestine, preventing undesirable autoimmune disease targeting CMA-expressing intestinal epithelium.

diseases associated with significant mortality. The persistent expression of GCC by metastatic tumors arising in the intestine but not by normal extraintestinal tissues makes GCC a sensitive and specific molecular marker for identifying primary malignancies of the upper GI tract and metastatic GI tumors, as well as staging patients with GI tumors, a critical prognostic determinant of patient management and survival. Indeed, the examination of stage B colorectal cancer patients, presumably cured by surgery, revealed an association between GCC mRNA in lymph nodes and disease recurrence—GCC mRNA was detectable in the lymph nodes of all patients who developed recurrent disease but not in any patient who remained free of disease for >5 years following surgery.<sup>21</sup> Ongoing prospective randomized multicenter clinical trials are exploring the utility of GCC quantitative RT-PCR for staging colorectal cancer patients and for detecting recurrent disease during postoperative surveillance. Uniform overexpression by colorectal tumors and novel ectopic expression by tumors of the esophagus and stomach underscore the potential utility of GCC as a therapeutic target in managing patients with GI malignancies.

#### GCC immunogenicity and antitumor immunity

Anatomical and immunological compartmentalization suggests that CMAs may serve as ideal immunotherapeutic target

antigens for derivative tumors. In the context of its near-universal overexpression by colorectal cancer cells, this hypothesis has been tested directly by employing GCC as a target antigen in preclinical animal models (A. Snook, B. Stafford, P. Li, L. Huang, R. Birbe, S. Schulz *et al.*, unpublished data). GCC-based viral vector immunization protected against challenge with GCC-expressing colorectal cancer cells, reducing tumor growth ~90% and extending survival 75% in mice with subcutaneous isografts. GCC-specific immunization also reduced the number of metastatic nodules and reduced tumor burden ~90% in mice with liver metastases. Moreover, immunization reduced tumor burden ~80%, quantified by <sup>18</sup>F-fluorodeoxyglucose positron emission tomography and nodule enumeration, and extended the survival of mice ~50% with lung metastases (Figure 3). GCC-targeted antitumor immunity is mediated by unique differential lineage-specific immune cell responses following viral vector immunization, which included CD8<sup>+</sup> T cells, consistent with their generally accepted principal contribution to antitumor immunity, but not CD4<sup>+</sup> T or B cells. Consistent with other reports of absent mucosal immune responses following central compartment immunization,<sup>9,10,22</sup> GCC immunization did not induce intestinal autoimmune disease, following even multiple sequential prime-boost immunizations.



**Figure 3** GCC-specific antitumor immunity. Following control or GCC-based immunization, mice were challenged with GCC-expressing colorectal cancer cells to establish lung metastases. Disease was visualized in lungs by micro-computer-assisted tomography (microCT) and <sup>18</sup>F-fluorodeoxyglucose uptake positron emission tomography (PET). (a) Pronounced disease was detectable in mice immunized with the control vector, (b) whereas tumor burden was nearly eliminated in mice receiving GCC-based immunization. Lungs are indicated by dashed outline for clarity.



These results, employing GCC-specific immunization in preclinical models, provide proof-of-principle of the antitumor efficacy of CMA-targeted immunity. Immunization with GCC elicited identical immune responses in two mouse strains and provided effective antitumor immunity in multiple models of metastatic colorectal cancer, consistent with hypotheses of immunotherapy based on compartmentalization. Thus, immune compartmentalization provides a previously unrecognized opportunity for central compartment immunization against antigens selectively expressed in mucosae, providing effective therapeutic immunity against derivative tumors expressing CMAs. In that respect, unlike CT antigens, which are limited by heterogeneous expression among patients and tumors, CMAs are universally associated with mucosally derived malignancies. Thus CMAs, including GCC and potentially others like Cd2 and sucrose isomaltase, may offer a unique opportunity for effective antitumor immunity targeting self-antigens universally associated with disease.

## CONCLUSION

Cancer immunotherapy continues to be limited by a paucity of antigens that are universally associated with disease, strongly immunogenic, and cancer specific.<sup>2</sup> Although mutated self-antigens and CT antigens are generally strongly immunogenic, their use is limited by heterogeneity of expression. CMAs, normal mucosal antigens expressed throughout disease progression, are universally associated with disease and cancer specific within the central immune compartment. Employing GCC as a model antigen revealed the immunogenicity of CMAs and the efficacy of CMA-targeted immunotherapy against metastatic colorectal cancer. In the clinical setting, CMA-targeted immunotherapy will likely have the greatest impact in patients with minimal residual disease at risk of developing recurrence (tumor, node and metastasis (TNM) stages I and II) or as adjuvant immunization in combination with fluoropyrimidine therapy for patients with regional metastases (TNM stage III). Ectopic expression of GCC, as well as other intestine-specific molecules, in esophageal and gastric carcinomas suggests potential immunotherapeutic applications in these diseases as well.

Although molecular mechanisms underlying immune compartmentalization beyond the GI tract remain poorly understood, CMA-directed immunotherapy may be applicable to malignancies derived from other mucosae,<sup>23,24</sup> such as oral, respiratory, mammary, and urogenital tissues, to treat head and neck, lung, breast, and bladder cancers, respectively. The identification and translation of CMAs may contribute to the long-awaited solution to antigen-specific immunotherapy for cancer.

## ACKNOWLEDGMENTS

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## CONFLICT OF INTEREST

Targeted Diagnostics and Therapeutics, Inc., in part, supported this research and hold a license to patents that are directly applicable to this work.

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# EXHIBIT B

# **Guanylyl cyclase C -induced immunotherapeutic responses opposing tumor metastases without autoimmunity**

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**ABBREVIATIONS:** GCC, guanylyl cyclase C; AV, adenovirus; RV, rabies virus; VV,

vaccinia virus; ELISA, enzyme-linked immunosorbent assay; ELISpot, enzyme-linked immunospot assay; SF, spot-forming; SFC, spot-forming cell; 95% CI, 95% confidence interval.

Title:	11 words
Abstract:	306 words
Pages:	40
Words:	3025 words
References:	46
Figures:	7
Tables:	1
Supplemental Figures:	1
Supplemental Tables:	1

## ABSTRACT

**Background:** One of the greatest impediments to cancer immunotherapy is the paucity of antigens that are tumor-specific, sufficiently immunogenic, and shared among patients. Mucosa-restricted antigens that are expressed by tumor cells represent a novel class of vaccine targets exploiting immunologic privilege, which limits systemic tolerance to those antigens, and immunologic partitioning, which shields mucosae from systemic autoimmune responses. Here we defined the immunogenicity and antitumor efficacy of guanylyl cyclase C (GCC), a protein that is normally restricted to intestinal mucosa and universally expressed by metastatic colorectal cancer.

**Methods:** BALB/c mice (n=197) were immunized with recombinant viral vectors before (prophylactic) or following (therapeutic) a lethal challenge of GCC-expressing mouse colon cancer cells, and antitumor efficacy was monitored by quantifying metastasis and survival. Induction of autoimmunity was monitored by histopathology. Induction of GCC-specific B cell and CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were determined by enzyme-linked immunosorbent assay (ELISA) and ELISpot, respectively. Tolerance to GCC was quantified by comparing responses in GCC-deficient (n=45) and wild-type (n=69) C57BL/6 mice. Statistical tests were two-sided.

**Results:** Immunization with GCC-expressing viral vectors opposed the formation of nascent metastases to liver (control vs GCC: mean = 30.4 vs 3.55 nodules, difference = 26.9 nodules, 95% confidence interval [CI] = 8.47 to 45.3 nodules; *P*

= .008) and lung (control vs GCC: mean =263 vs 55.7 nodules, difference = 207, 95% CI = 163 to 251;  $P < .001$ ) and extended the median survival of mice with established lung metastases following therapeutic immunization (control vs GCC: 29 vs 38 days,  $P = .024$ ), without autoimmunity. Antitumor efficacy reflected asymmetric tolerance that was characterized by CD8<sup>+</sup> T cell, but not CD4<sup>+</sup> T cell or antibody, responses.

**Conclusions:** Immunologic partitioning, which prevents autoimmunity, together with immunologic privilege, which promotes systemic immune responses, highlight the potential of mucosa-restricted antigens, particularly GCC, as therapeutic targets for metastatic cancer.

One of the greatest impediments to cancer immunotherapy is the paucity of antigens that are tumor-specific, sufficiently immunogenic, and shared among patients (1). In lieu of ideal targets, antitumor immune responses are generally directed to tissue-specific, rather than tumor-specific proteins. Barriers to using self-antigens include the potential development of concomitant autoimmunity and tolerance, which limits immunotherapeutic efficacy (2). Attempts to circumvent these limitations have included the use of self-proteins that are expressed in immunologically-privileged compartments, for example, cancer testis antigens (3). Their ectopic expression in tumors outside the restricted compartments provides opportunities for targeted immunologic responses that are essentially directed to tumor-specific antigens. The best characterized tumor-associated antigens derived from privileged compartments include cancer testis antigens (3), which represent more than 40 gene products, including MAGE and NY-ESO, whose expression is normally restricted to testis but which are anomalously expressed by cancers. Although many cancer testis antigens exhibit characteristics that are suited to immunologic targeting in cancer, their use is restricted by heterogeneity of expression in tumors. With the exception of melanoma and lung cancers, individual cancer testis antigens are expressed in less than 20% of epithelial tumors, and colorectal tumors exhibit the lowest frequency of expression (4), rendering them suboptimal targets in that disease and limiting their clinical use. An unexplored variation on this theme exploits the



universal expression of mucosa-restricted antigens by derivative tumors, in the context of the established asymmetry in immunologic cross-talk between mucosal and systemic compartments (5). This asymmetry offers a unique advantage reflecting the intersection of immunologic privilege and immunologic partitioning. Indeed, immunologic privilege limits systemic tolerance to mucosal antigens and facilitates therapeutic antitumor responses, whereas immunologic partitioning shields mucosae from systemic immune responses and limits autoimmunity (6-9).

Guanylyl cyclase C (GCC), the receptor for diarrheagenic bacterial heat-stable enterotoxins and the endogenous paracrine hormones guanylin and uroguanylin (10), is expressed in apical membranes of intestinal epithelial cells, restricting it to mucosal immune compartments (11-14). Moreover, GCC is universally expressed by primary and metastatic colorectal tumors (11-13, 15, 16). This pattern of expression suggests that GCC may qualify as an efficacious mucosa-restricted immunotherapeutic target in colorectal cancer, which is the second leading cause of cancer mortality in the United States and the fourth most common worldwide (17). Here, we define the immunogenicity and therapeutic utility of GCC in mouse models of metastatic colorectal cancer.

## **MATERIALS AND METHODS**

**Guanylyl Cyclase Alignment.** Mouse GCA (Genbank Accession number NP\_032753), GCB (NP\_776149), GCC (NP\_659504), GCE (NP\_032218.2),

GCF (NP\_001007577), GCG (NP\_001074545.1), and NPR3 (NP\_001034270.1) were aligned using the CLUSTAL W algorithm (18).

**Mice.** C57BL/6 (n=30) and BALB/c mice (n=197) were obtained from the NCI Animal Production Program (Frederick, MD). GCC-deficient (GCC<sup>-/-</sup>) mice (19) were backcrossed with C57BL/6 mice for more than 10 generations to produce GCC<sup>-/-</sup> congenic C57BL/6 mice and wild-type (GCC<sup>+/+</sup>) littermates (20). Mouse protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee.

**Plasmids.** A truncated GCC<sub>1-430</sub> construct (GCC<sub>ECD</sub>) containing the extracellular ligand binding domain and a C-terminus hexahistidine tag was generated by polymerase chain reaction (PCR) using GCC cDNA and subcloned to produce recombinant viruses. The membrane-bound recombinant GCC<sub>1-461</sub> construct, GCC<sub>TM</sub>, was produced by PCR using the extracellular ligand binding domain (residues 23–430) containing an N-terminus pentahistidine tag. The N-terminus signal peptide (residues 1–23) and C-terminus transmembrane domain (residues 431–461) containing a C-terminus hexahistidine tag were then added by PCR and subcloned into pMSCV2.2-Puro to generate the plasmid GCC<sub>TM</sub>-pMSCV2.2-Puro.

**Recombinant viruses.** Replication-deficient human type 5 recombinant adenovirus was generated using the ViraPower Adenoviral Expression System and plasmid pAd/CMV/V5-DEST (Invitrogen, Carlsbad, California). Adenovirus was purified using the Adeno-X Virus Purification Kit (Clontech, Mountain View,

California) and titered using the Adeno-X Rapid Titer Kit (Clontech). LacZ-AV was acquired from Clontech (LacZ-Adeno-X), expanded, purified, and titered in parallel with GCC<sub>ECD</sub>-AV. GCC<sub>ECD</sub>-RV and control RV were generated using the BsiWI/Nhe restriction sites in the SPBN rabies virus backbone, and infectious virus was recovered as described previously (21). The control RV (RV-NP/SIIN) was described previously (22). GCC<sub>ECD</sub>-VV was generated using the pSC11 vaccinia plasmid (23). The control VV (VV-1686) was described previously (24). Mice were immunized with  $1 \times 10^8$  IFU of adenovirus or  $1 \times 10^7$  FFU of rabies virus by intramuscular injection of the anterior tibialis or with  $1 \times 10^7$  PFU vaccinia virus by intraperitoneal injection (n=322 AV, n=10 AV and VV, n=58 AV, RV and VV).

**Cell lines.** C57BL/6-derived MC38 colorectal cancer cells were provided by Jeffrey Schlom (National Cancer Institute, National Institutes of Health, Bethesda, MD). BALB/c-derived CT26 colorectal cancer cells were from the American Type Culture Collection (Manassas, Virginia). Both cell lines lack endogenous GCC expression, as determined by radiolabeled-ligand binding and quantitative reverse-transcriptase (qRT)-PCR. Stable CT26 cell lines were generated by transducing CT26 cells with retrovirus produced from 293T cells that were transiently transfected with pCL-Ampho (Imgenex, San Diego, California) and GCC<sub>TM</sub>-pMSCV2.2-Puro, followed by antibiotic selection. GCC<sub>TM</sub> expression was quantified by FACS analysis after staining for the extracellular pentahistidine on GCC<sub>TM</sub> using mouse monoclonal anti-pentahistidine-Alexa 488 antibody (Qiagen, Valencia, California) and by binding of radiolabeled heat-stable

enterotoxin (ST) to purified membranes (25).

**GCC<sub>ECD</sub> Protein Purification.** Hexahistidine-tagged GCC<sub>ECD</sub> (GCC<sub>ECD</sub>-6xHis) was purified from supernatants of 293A cells that had been transduced with GCC<sub>ECD</sub>-AV using Ni-NTA agarose beads (Qiagen). A baculovirus expression system was used to produce a hexahistidine-tagged signal peptide-deficient GCC extracellular domain protein (GCC<sub>23-430</sub>) using the transfer vector pVL1393 and the Sapphire Baculovirus DNA Kit (Orbigen, San Diego, California). Baculovirus-produced GCC<sub>ECD</sub>-6xHis was purified using Ni-NTA agarose beads (Qiagen). 293A-expressed GCC<sub>ECD</sub> was used in enzyme-linked immunosorbent assays (ELISAs), and baculovirus-expressed GCC<sub>ECD</sub> was used in CD4<sup>+</sup> T cell assays.

**Antigen-Specific IgG Antibody Detection by ELISA.** BALB/c were naïve (n=4) or immunized with control AV (n=4) or GCC<sub>ECD</sub>-AV (n=4) and GCC<sup>+/+</sup> (+/+) and GCC<sup>-/-</sup> (-/-) C57BL/6 were immunized with control AV (n=4 +/+ and n=4 -/-), GCC<sub>ECD</sub>-AV (n=4 +/+ and n=4 -/-) or with an irrelevant GST-fusion protein as a negative control (n=3 +/+ and n=3 -/-). Mice were killed by CO<sub>2</sub> asphyxiation and serum was collected 10-14 days after immunization. Immunosorbent plates (Nunc, Rochester, New York) were coated with purified GCC<sub>ECD</sub>-6xHis at 10 µg/mL or with irrelevant adenoviral particles at 1x10<sup>7</sup> IFU/mL to detect GCC<sub>ECD</sub>-specific or AV-specific responses, respectively. Coated plates were incubated with serum or with mouse anti-pentahistidine IgG (Qiagen) as a positive control. Specific antibodies were detected with HRP-conjugated goat anti-mouse

immunoglobulin (Jackson Laboratories, Bar Harbor, Maine) and ABTS substrate (Pierce, Rockford, Illinois). Data represent the mean absorbance at 405 nm of individual mice.

**Antigen-Specific T cell Response Detection by IFN $\gamma$  ELISpot.** BALB/c were immunized with control AV (n=16) or GCC<sub>ECD</sub>-AV (n=16) and GCC<sup>+/+</sup> (+/+) and GCC<sup>-/-</sup> (-/-) C57BL/6 were immunized with control AV (n=24 +/+ and n=12 -/-) or GCC<sub>ECD</sub>-AV (n=32 +/+ and n=20 -/-). Mice were killed by CO<sub>2</sub> asphyxiation and spleens were collected 10 days after immunization. Animals were assayed by pooling spleens from 2 mice per experiment and assaying in triplicate. Data are representative of 2-6 independent experiments, as indicated in figure legends. Multiscreen filtration plates (Millipore, Billerica, Massachusetts) were coated with anti-mouse interferon gamma (IFN $\gamma$ )-capture antibody (BD Pharmingen, San Jose, California). To assay CD8<sup>+</sup> T cell responses, splenocytes or CD8<sup>+</sup> T cells that had been separated from splenocytes by magnetic activated cell sorting (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) were plated at 10,000–250,000 cells per well and incubated with stimulator cells (MC38 for C57BL/6 mice or CT26 for BALB/c mice). To measure GCC<sub>ECD</sub> or  $\beta$ -galactosidase-specific responses, stimulator cells were transduced with replication-deficient GCC<sub>ECD</sub>- or LacZ-adenovirus, respectively, at a multiplicity of infection (MOI) of 500 (MC38) or 100 (CT26) and treated with 1000 U/mL recombinant mouse IFN $\gamma$  (EMD Biosciences, San Diego, California) for 48 hours to increase MHC expression. To assay CD4<sup>+</sup> T cell responses, MACS-sorted CD4<sup>+</sup> T cells (Miltenyi Biotec)

were mixed with naïve splenocytes as antigen presenting cells and incubated on antibody-coated plates with GCC<sub>ECD</sub>-6xHis (0-100 µg/mL) or irrelevant purified adenovirus particles (0-1x10<sup>8</sup> IFU/mL), to measure GCC<sub>ECD</sub>-specific or AV-specific responses, respectively. After 24–28 hours of stimulation, cells were removed by washing and spots were developed with biotinylated anti-IFN $\gamma$  detection antibody (BD Pharmingen) and alkaline phosphatase-conjugated streptavidin (Pierce), followed by NBT/BCIP substrate (Pierce). Spot numbers were quantified using computer-assisted video imaging analysis (ImmunoSpot v3, Cellular Technology, Shaker Heights, Ohio). Data are reported as spots (IFN $\gamma$ -producing cells) per 10<sup>6</sup> cells or per well.

**GCC Immunization against Subcutaneous Colon Tumors.** BALB/c mice received a prophylactic immunization of AV (n=5 control and GCC<sub>ECD</sub>), AV and VV at 28 day intervals (n=5 control and GCC<sub>ECD</sub>), or AV, RV and VV at 28 day intervals (n=7-8 control and GCC<sub>ECD</sub>). Subcutaneous tumors were then established on the flanks of mice with 1x10<sup>5</sup> CT26-GCC<sub>TM</sub> cells 1 week after final immunization and tumor growth was quantified twice/week for 45 days by measuring three orthogonal diameters and calculating volumes using:  $\frac{4}{3}\pi \cdot r_1 \cdot r_2 \cdot r_3$ , in which (r) is half the diameter. For survival analysis, mice were killed by CO<sub>2</sub> asphyxiation when tumors achieved a volume of 1200 mm<sup>3</sup>, a surrogate end-point used in compliance with institutional animal care guidelines.

**GCC Immunization against Colon Cancer Metastases to Liver.**

BALB/c mice were immunized with control AV (n=11) or GCC<sub>ECD</sub>-AV (n=12). Liver metastases were established by injecting  $1 \times 10^5$  cells into the exteriorized spleens of mice 7 days after immunization (26). Mice were killed by CO<sub>2</sub> asphyxiation and metastases were counted in excised livers 21 days after tumor challenge.

#### **GCC Immunization against Colon Cancer Metastases to Lung.**

To examine prophylaxis, BALB/c mice were immunized with control AV (n=21) or GCC<sub>ECD</sub>-AV (n=31) and lung metastases were established by injecting  $5 \times 10^5$  CT26-GCC<sub>TM</sub> cells into the tail veins of mice 7 days after immunization. Some mice were imaged by positron emission tomography/micro computer tomography (PET/microCT) 14 days later, then killed by CO<sub>2</sub> asphyxiation, and metastasis were counted (27). In other mice, survival was monitored daily for 45 days and recorded. In studies of therapeutic immunization, lung metastases were established in BALB/c mice (n=39) by tail vein injection with  $1 \times 10^5$  CT26-GCC<sub>TM</sub> cells. Mice were then immunized with control (n=22) or GCC<sub>ECD</sub> (n=17) AV on day 3, followed by sequential boosting with RV and VV every 12 to 26 days such that most mice received all three immunizations before dying from metastasis growth. Survival was monitored daily for 55 days and recorded.

**Positron Emission Tomography/Micro Computer Tomography Metastasis Quantification.** Mice were immunized with control AV (n=5) or GCC<sub>ECD</sub>-AV (n=9) 7 days before being injected by tail vein with  $5 \times 10^5$  GCC<sub>TM</sub> cells. Fourteen days later, mice received 0.45 mCi <sup>18</sup>F-fluorodeoxyglucose by tail vein, and positron

emission tomography (PET) images were collected 2 hours later on a Mosaic scanner (Philips Medical Systems, Andover, Massachusetts). Computer tomography (CT) images were acquired on a microCAT II (Imtek, Inc, Knoxville, Tennessee). For quantification of  $^{18}\text{F}$ -fluorodeoxyglucose uptake and, consequently, tumor burden, a volumetric region of interest was defined in the lungs. Using calibration parameters that were derived from a cylinder of known size and activity, absolute quantification values of % injected dose per g of tissue (% ID/g) were calculated. Average lung values were normalized to uptake in liver within each mouse (lung minus liver).

**Detection of Serum Anti-Nuclear Antibodies.** Anti-nuclear antibodies were quantified in serum using an anti-ANA ELISA Kit (Alpha Diagnostics, San Antonio, Texas) in C57BL/6 mice 10 days after immunization with LacZ-AV (n=6) or GCC<sub>ECD</sub>-AV (n=6). Serum from 8-week-old male MRL/MpJ-Fas<sup>lpr</sup>/J mice (Jackson Laboratories) (n=3) served as positive controls and serum from naïve mice (n=2) served as negative controls. Anti-nuclear IgG was quantified using a standard curve and reported as  $\mu\text{g IgG/mL}$ .

**Autoimmune Pathology.** Chemistry profiles were commercially determined (Charles River Laboratories, Wilmington, Massachusetts) on fresh serum from previously immunized GCC<sup>+/+</sup> (n=5 control AV and n=6 GCC<sub>ECD</sub>-AV) and GCC<sup>-/-</sup> (n=6 control AV and n=5 GCC<sub>ECD</sub>-AV) mice. Tissues from immunized BALB/c mice (n=3 for each immunization) were formalin-fixed, paraffin-embedded, stained by hematoxylin & eosin, blindly labeled, and analyzed by a pathologist



(RB).

**Statistical Analyses.** Cohort sizes were selected to provide 80% power to detect 2-fold differences between groups. Survival analysis used Mantel-Haenszel log-rank test (GraphPad Prism Software, San Diego, California). Two-way ANOVA (Prism) was used for subcutaneous tumor growth studies, antigen-specific antibody assays and T cell assays. Student's *t* test or Welch's *t* test (for tests with unequal intergroup variances) were used for all other statistical analyses. All statistical tests were two-sided, except for liver weight analysis, for which a one-sided test was appropriate. *P* values less than .05 were considered statistically significant.

## RESULTS

*Vaccine Strategy.* The seven members of the particulate guanylyl cyclase family have a canonical structure with highly homologous cytoplasmic catalytic and regulatory domains and extracellular domains possessing minimal sequence homology, reflecting the diversity of their cognate peptide ligands (14) (Figure 1, A). Conservation of cytoplasmic domains among isoforms (Figure 1, B) and the broad tissue distribution of guanylyl cyclases A, B, and G identified the extracellular ligand-binding domain of GCC as the only unique segment of the molecule, therefore, focusing vaccine generation on only the GCC extracellular ligand-binding domain. Because none of the established mouse cell lines express endogenous GCC, CT26 mouse colon cancer cells were retrovirally transduced to stably express a recombinant mouse GCC (Figure 1, C) that was

truncated beyond the transmembrane domain and lacked intracellular domains (GCC<sub>1-461</sub>; GCC<sub>TM</sub>; Figure 1, B) at levels within the range of normal murine enterocytes and human colon cancer cells (Figure 1, D). Viral vaccine vectors were constructed to express a secreted form of GCC that was truncated beyond the ligand binding domain and lacked transmembrane and intracellular domains (GCC<sub>1-430</sub>; GCC<sub>ECD</sub>; Figure 1, B).

*Prophylactic GCC immunotherapy against subcutaneous colon tumors.* BALB/c mice received a prophylactic immunization comprising an escalating heterologous prime boost regimen of replication-deficient recombinant adenovirus (AV), recombinant attenuated rabies virus (RV), and recombinant vaccinia virus (VV), with 28 days between each immunization. Seven days after the last immunization, mice were subcutaneously injected with  $1 \times 10^5$  CT26 mouse colon carcinoma cells expressing GCC<sub>TM</sub> (CT26-GCC<sub>TM</sub> cells), and tumor volume was monitored for 21 days. A single immunization with GCC<sub>ECD</sub>-AV did not alter the growth of CT26-GCC<sub>TM</sub> cells compared with a control AV immunization (Figure 2, A). However, a prophylactic heterologous prime-boost strategy using GCC<sub>ECD</sub>-AV followed by GCC<sub>ECD</sub>-VV reduced tumor growth (Figure 2, B; mean day 21 tumor volume: control vs GCC<sub>ECD</sub>, 449 mm<sup>3</sup> vs 124 mm<sup>3</sup>, difference = 325 mm<sup>3</sup>, 95% CI = 202 to 448 mm<sup>3</sup>;  $P < .001$ ). Moreover, GCC<sub>ECD</sub>-AV followed sequentially by GCC<sub>ECD</sub>-RV and GCC<sub>ECD</sub>-VV reduced tumor growth (Figure 2, C and D; mean day 21 tumor volume: control vs GCC<sub>ECD</sub>, 526 mm<sup>3</sup> vs 29 mm<sup>3</sup>, difference = 487 mm<sup>3</sup>, 95% CI = 337 to 656 mm<sup>3</sup>,

$P < .001$ ). Further, both the AV-VV and AV-RV-VV heterologous prime boost regimens extended survival of GCC<sub>ECD</sub>-immunized mice compared with control immunization (Figure 2, E; median survival in control AV-VV vs GCC<sub>ECD</sub> AV-VV, 28 days vs 33 days;  $P = .018$ , and Figure 2, F; control AV-RV-VV vs GCC<sub>ECD</sub> AV-RV-VV, 24 days vs 42 days;  $P < .001$ ).

*Prophylactic and therapeutic GCC immunotherapy against parenchymal colon cancer metastases.* More than 50% of patients with colorectal cancer die of metastatic disease, primarily in liver and lung (5). To mimic human disease in parenchymal metastasis models, mice were administered  $1 \times 10^5$  CT26-GCC<sub>TM</sub> cells by intrasplenic injection to establish liver metastases 7 days after immunization with GCC<sub>ECD</sub>-expressing or control AV. Three weeks later, livers were collected and tumor burden was quantified by counting nodules and measuring liver wet weight, a marker of metastatic disease. In contrast to efficacy against subcutaneous tumor growth, immunization with GCC<sub>ECD</sub>-AV alone reduced the formation of liver nodules by approximately 90% (Figure 3, A and C; control AV vs GCC<sub>ECD</sub>-AV: 30.4 vs 3.55 nodules, difference = 26.9 nodules, 95% CI = 8.47 to 45.3 nodules;  $P = .008$ ) and liver wet weight by 25% (Figure 3, B; control AV vs GCC<sub>ECD</sub>-AV: 1.86 g vs 1.44 g, difference = 0.42 g, 95% CI = -0.018 g to 0.948 g;  $P = .044$ , one-sided Student's *t* test). In addition, immunization with GCC<sub>ECD</sub>-AV 7 days before intravenous seeding of lung metastases with  $5 \times 10^5$  CT26-GCC<sub>TM</sub> cells reduced the formation of lung nodules by approximately 80% (Figure 4, C and D; control AV vs GCC<sub>ECD</sub>-AV: 263 vs 56

nodules, difference = 207 nodules, 95% CI = 163 to 251 nodules:  $P < .001$ ) and tumor burden by approximately 80%, as quantified by  $^{18}\text{F}$ -fluorodeoxyglucose positron emission tomography (PET) (Figure 4, A and B; relative uptake in control AV vs GCC<sub>ECD</sub>-AV: 1.27 vs 0.25, difference = 1.02, 95% CI = 0.71 to 1.32;  $P < .001$ ). Furthermore, GCC<sub>ECD</sub>-AV immunization extended survival of mice bearing lung metastases by approximately 50% (Figure 4, E; median survival in control AV vs GCC<sub>ECD</sub> AV, 23 days vs 35 days;  $P < .001$ ). Moreover, beyond prophylaxis, GCC-directed immunization was efficacious in treating established parenchymal tumor metastases because sequential immunization with GCC<sub>ECD</sub>-AV, -RV, and -VV in mice carrying lung metastases extended median survival (Figure 4, F; control AV-RV-VV vs GCC<sub>ECD</sub> AV-RV-VV, 29 days vs 38 days;  $P = .024$ ), with an immunotherapeutic efficacy comparable to self-antigens that have advanced from mouse models to human trials (28, 29).

*CD8<sup>+</sup> T cells, but not CD4<sup>+</sup> T cells or antibodies as mediators of GCC-targeted immunity.* Protection against metastatic tumor cells by immunization with recombinant viruses presumably reflects mucosal compartmentalization of GCC expression, resulting in incomplete systemic tolerance and selected immune cell responses to that antigen. Immunization of BALB/c mice with GCC<sub>ECD</sub>- or LacZ-AV elicited AV-specific, but not GCC- specific, IgG responses (Figure 5, A and B). Similarly, AV- specific, but not GCC-specific, Th1 CD4<sup>+</sup> T cell responses were induced in immunized mice (Figure 5, C and D). In contrast, antigen-specific CD8<sup>+</sup> T cell responses directed against  $\beta$ -galactosidase (LacZ), a foreign

antigen, or GCC were nearly equivalent (Figure 5, E and F; mean specific spots per well at 250,000 effectors: control vs GCC<sub>ECD</sub>, 49.3 in 33.0, difference = 16.3, 95% CI = 2.41 to 30.3;  $P = .086$ ). Inhibition of parenchymal metastases that are associated with enhanced survival in the context of CD8<sup>+</sup> T cell responses, but not CD4<sup>+</sup> T or B cell responses, induced by GCC immunization suggests that antitumor immunity is CD8<sup>+</sup> T cell-mediated and underscores the central role of CD8<sup>+</sup> T lymphocytes as key antitumor effectors of the adaptive immune system.

*Lineage-specific systemic tolerance to GCC.* CD8<sup>+</sup> T cell responses, but not CD4<sup>+</sup> T or B cell responses, to GCC suggest that mucosa-restricted expression induces selective systemic tolerance reflecting structural characteristics of GCC influencing antigenic competence and/or compartmentalization (30, 31).

Tolerance was explored by comparing responses of wild-type (GCC<sup>+/+</sup>) C57BL/6 mice with GCC<sup>-/-</sup> mice, in which GCC was eliminated (19). GCC<sup>+/+</sup> and GCC<sup>-/-</sup> mice that were immunized with GCC<sub>ECD</sub>-AV or LacZ-AV produced identical adenovirus-specific antibody responses (Figure 6, A), Th1 CD4<sup>+</sup> T cell responses (Figure 6, C), and  $\beta$ -galactosidase-specific CD8<sup>+</sup> T cell responses (Figure 6, G), suggesting that GCC-deficiency does not broadly alter antigen-specific immune responses. However, GCC<sup>-/-</sup> mice but not GCC<sup>+/+</sup> mice produced antibodies (Figure 6, B) and Th1 CD4<sup>+</sup> T cells (Figure 6, D) that were directed against GCC<sub>ECD</sub>. In contrast, GCC<sup>+/+</sup> C57BL/6 mice, like GCC<sup>+/+</sup> BALB/c mice (see Figure 5, E and F), produced CD8<sup>+</sup> T cell responses that specifically recognized  $\beta$ -galactosidase or GCC<sub>ECD</sub> following immunization with LacZ-AV or GCC<sub>ECD</sub>-AV,

respectively (Figure 6, E and F). However, although CD8<sup>+</sup> T cell responses to  $\beta$ -galactosidase were comparable in GCC<sup>-/-</sup> mice following immunization with LacZ-AV (Figure 6, G), responses to GCC were attenuated in GCC<sup>+/+</sup> mice compared with GCC<sup>-/-</sup> mice following immunization with GCC<sub>ECD</sub>-AV (Figure 6, H). Robust GCC-directed antibody responses and CD4<sup>+</sup> T cell responses in GCC<sup>-/-</sup> mice following immunization with GCC<sub>ECD</sub>-AV underscore the antigenic competence of GCC to induce responses in all arms of the adaptive immune system. In contrast, incomplete tolerance to GCC in wild-type BALB/c and C57BL/6 mice reflects mucosal partitioning and restricted cross-compartmental antigen availability (30, 31).

*Antitumor immunity without collateral autoimmune disease.* The established restriction of immunologic crosstalk between systemic and mucosal compartments in conjunction with systemic tolerance in CD4<sup>+</sup> T cell responses to mucosa-restricted antigens identified herein should limit autoimmune disease in response to GCC-based immunization. Mice that were serially immunized with three viruses encoding GCC<sub>ECD</sub> and exhibiting maximum antitumor responses (see Figure 2, C, D, and F) were healthy, with no signs or symptoms of inflammatory bowel disease, including weight loss, failure to thrive, altered bowel habits, or rectal bleeding. Similarly, GCC<sup>+/+</sup> and GCC<sup>-/-</sup> mice that were immunized with GCC<sub>ECD</sub>-AV exhibited normal organ and metabolic function, as quantified by serum chemistries (Table 1). Further, autoimmune-mediated tissue damage, which was quantified by serum anti-nuclear antibodies, was absent in

GCC<sup>+/+</sup> mice that were immunized with GCC<sub>ECD</sub>-AV (Figure 7, A). Moreover, the gastrointestinal tract, lungs, liver, and kidneys of mice that were serially immunized with three viruses encoding GCC<sub>ECD</sub> exhibiting maximum antitumor responses were free of damage and immune cell infiltration (Figure 7, B and Supplementary Table 1, available online). In that context, GCC is a cancer mucosa antigen yielding efficacious immunotherapeutic antitumor responses without autoimmune tissue damage, underscoring the potential for mucosa-restricted proteins as vaccine targets for derivative metastatic tumors.

## DISCUSSION

Here we have identified GCC as the first in a newly defined category of self-antigens, cancer mucosa antigens, that are expressed normally in the immunologically privileged mucosa and by tumors (5). Viral vector immunization with GCC elicited systemic CD8<sup>+</sup> T cell responses in both strains of mice tested. Moreover, these responses effectively opposed tumor growth in multiple prophylaxis models of GCC-expressing metastatic colorectal cancer, and in a model of immunotherapy of established parenchymal metastases. Comparison of responses in GCC<sup>+/+</sup> and GCC<sup>-/-</sup> mice revealed CD4<sup>+</sup> T cell and antibody responses in GCC<sup>-/-</sup>, but not in two strains of GCC<sup>+/+</sup> mice, suggesting that tolerance to cancer mucosa antigens may be universally characterized by absent CD4<sup>+</sup> T cell and antibody responses but only partially attenuated CD8<sup>+</sup> T cell responses, making them attractive targets for tumor immunotherapy. Histological

examination of tissues and measurement of serum markers of autoimmunity and tissue damage in mice with effective concomitant antitumor immunity revealed an absence of adverse effects induced by GCC immunotherapy.

In contrast to currently available cancer testis antigens, the universal expression of mucosa-restricted antigens by derivative tumors offers a unique solution to the application of self-antigens from immunologically privileged sites to tumor immunotherapy. This approach leverages the established immunologic partitioning of systemic and mucosal compartments (6-9). Asymmetry in signaling across compartments, wherein systemic immune responses rarely extend to mucosae, limits the risk of autoimmunity following systemic immunization. Conversely, expression that is restricted to mucosae limits antigen access to the systemic compartment and opposes tolerance to mucosal antigens and, therefore, may overcome the inherent limitations of immunotherapy directed to self antigens. However, systemic tolerance to mucosa-restricted antigens, which can limit antitumor efficacy, has been only incompletely defined. Here, immunization revealed lineage-specific T cell tolerance in the systemic compartment, which contained CD8<sup>+</sup> T cell responses, but not CD4<sup>+</sup> T cell responses, to GCC. Tolerance reflects thymic and/or peripheral mechanisms, rather than antigenicity, because GCC<sup>-/-</sup> mice responded to GCC in all arms of the adaptive immune system, whereas in two strains of GCC<sup>+/+</sup> mice, GCC elicited only CD8<sup>+</sup> T cell responses. Incomplete central tolerance to GCC presumably reflects anatomical, functional, and immunologic



compartmentalization wherein sequestration of mucosal antigens provides insufficient antigen for complete systemic tolerance (30, 31).

In that context, common molecular mechanisms mediating incomplete systemic tolerance to mucosal antigens have not yet emerged. Indeed, tolerance to the gastric-specific H<sup>+</sup>/K<sup>+</sup> ATPase is mediated peripherally (32) and that to a mucosa-selective carcinoembryonic antigen transgene is mediated by the thymus (33). Here, incomplete tolerance may reflect CD4<sup>+</sup> T cell-independent induction of antigen-specific CD8<sup>+</sup> T cell responses (34). Alternatively, virus-specific CD4<sup>+</sup> T cells may provide sufficient support to develop efficacious GCC-specific CD8<sup>+</sup> T cell responses (35). Incomplete tolerance may be mediated, in part, by AIRE-regulated peripheral antigen expression within thymic epithelial cells, resulting in T cell deletion (36, 37). In addition, intestinal antigens may be acquired by mucosal dendritic cells and transported to mesenteric lymph nodes, where they induce regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells, deletion/anergy of naïve T cells, or other mechanisms that diminish immune responsiveness to GCC (38, 39). Although mechanisms that mediate incomplete systemic tolerance remain to be defined, these advantageous immune cell responses underscore the potential of cancer mucosa antigens as immunotherapeutic targets to prevent tumor metastases in the absence of collateral autoimmune tissue damage.

GCC immunization produced effective antitumor immune responses likely mediated by CD8<sup>+</sup> T cells in the systemic compartment in the absence of mucosal autoimmune disease, reflecting compartment-restricted lymphocyte

recirculation imprinted by tissue-specific activation (6, 7, 40-43). T cells that are activated in mesenteric lymph nodes home to lymphoid structures that are associated with the gut wall, including the lamina propria and Peyer's patches, and those that are activated systemically home primarily to the spleen and to peripheral lymph nodes and associated tissues (41-44). Compartmentalized recirculation reciprocally increases the efficiency of regional immune responses while decreasing tissue antigen cross-reactivity (41). In that context, the functional independence of compartments is reflected by the paucity of mucosal responses to systemic immunization (6-9). Here, this functional independence, wherein systemic immune responses rarely extend to the mucosal compartment, has been exploited to generate therapeutic responses to metastatic cancer without collateral autoimmune disease by using a self-antigen whose expression is normally restricted to mucosa but is universally expressed in metastatic colorectal tumors.

In the scope of current clinical practice and management of patients with colorectal cancer, vaccination using cancer mucosa antigens generally, and GCC specifically, should have the greatest impact on survival in patients who are at risk for developing metastatic disease. Patients who are ostensibly free of regional metastases at the time of diagnosis and staging (TNM Stages I, II) are at substantial risk for developing metastatic disease, reflecting the presence of occult micrometastases (17). In this clinically heterogeneous population, some of whom have occult residual disease, adjuvant immunotherapy could reduce

recurrences and extend disease-free survival, reflecting maximum therapeutic efficacy in the context of minimal metastatic tumor burden. Similarly, cancer mucosa antigen vaccines could impact fluoropyrimidine-based adjuvant therapeutic regimens, which are the mainstay for patients with TNM Stage III disease with regional lymph node metastases (17). Further, GCC-based vaccines specifically could reduce mortality in patients with esophageal and gastric cancer, reflecting the role of intestinal metaplasia and the associated novel ectopic expression of that antigen in those malignancies (45). Moreover, the efficacy of adjuvant immunotherapy in gastrointestinal malignancies might benefit from polyvalent vaccines that incorporate cancer mucosa antigens other than GCC, for example Cdx2 and sucrase-isomaltase, which also are intestinally-restricted and highly expressed in mucosa-derived malignancies.

Beyond the GI tract, the present observations establish a framework for exploiting immunologic compartmentalization to achieve antimetastatic therapy in tumors that originate from other mucosae, including oral, respiratory, mammary, and urogenital for the treatment of cancers of the head and neck, lung, breast, vagina, and bladder, respectively. This potential for cancer mucosa antigens as immunotherapeutic targets highlights the gap in understanding mechanisms underlying systemic and peripheral tolerance and the segregation of adaptive immune responses across mucosae. Those considerations notwithstanding, the overarching principles of immunologic compartmentalization apply outside the gastrointestinal tract, wherein systemic immune responses do not extend to

extra-intestinal mucosal surfaces (8, 46). The established principles of immune partitioning in the context of the present results with GCC underscore the importance of defining the generalizability of cancer mucosa antigens as targets for immunotherapy of mucosa-derived tumors.

Limitations in this study relate to cell lines and mice as preclinical models of human malignancy. Cell lines may not accurately reflect the spectrum of antigen presentation or immunogenicity exhibited by human tumors. Also, the CT26 mouse tumor cell line used herein was genetically engineered to express GCC, albeit at levels comparable with those endogenously expressed in normal mouse intestinal cells and human tumor cells. Further, subcutaneous and parenchymal tumor metastasis models in mice may incompletely reflect the pathophysiology and immunology of metastatic disease in patients. Moreover, although observational data suggest the compartmentalization of mucosal and systemic immunity, its relevance and underlying mechanistic contribution to antitumor immunotherapy in humans are unknown. In addition, the immunogenicity of GCC in humans is not yet known. Finally, the generalizability of cancer mucosa antigens across different vaccine targets and mucosae for immunotherapy in animals and humans remains to be defined.

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## FIGURE LEGENDS

**Figure 1.** Vaccine design. **A)** Protein alignment of membrane-bound mouse guanylyl cyclases. Shaded boxes represent amino acid homology. Extracellular ligand-binding domains are nearly devoid of homology across the family. In

contrast, there is a high degree of homology within the intracellular domains of the different isoforms. For a full-size version of the alignment, see Supplementary Figure 1 (available online). **B)** GCC constructs used in these studies: GCC<sub>TM</sub>, which lacks cytosolic domains but includes the transmembrane domain and traffics to the cell surface; and GCC<sub>ECD</sub>, which lacks cytosolic and transmembrane domains and is secreted. GCC<sub>TM</sub> was used in tumor models and GCC<sub>ECD</sub> was used in viral vaccine vectors. **C–D)** Cell surface expression of GCC<sub>TM</sub> in transduced CT26 cells using anti-pentahistidine antibody and FACS analysis (C) and binding of <sup>125</sup>I-ST to membranes prepared from CT26-GCC<sub>TM</sub> cells (D). Membranes from C57BL/6 small intestine and colon, which endogenously express GCC, and T84 human colon cancer cells were used as positive controls. Binding data indicate means and upper 95% confidence intervals of triplicate measures, representing 2 independent experiments.

**Figure 2.** Prophylactic GCC-specific immunity against subcutaneous colon tumor progression. **A–C)** Growth of tumors in BALB/c mice that were prophylactically immunized with recombinant viruses expressing GCC<sub>ECD</sub> or control viruses using an escalating heterologous prime-boost strategy, including AV (A), AV followed by VV (B), or AV followed sequentially by RV and VV (C). Data are means of n=5–8 mice per immunization, and error bars indicate 95% confidence intervals (\*\*\*)  $P < .001$ , two-way ANOVA of tumor growth in control- vs GCC<sub>ECD</sub>-immunized mice). **D)** Images of control- and GCC<sub>ECD</sub> AV-RV-VV-immunized mice on day 24 with control tumors outlined for clarity. **E)** Survival analysis of mice from (B) ( $P =$

.018, two-sided Mantel-Haenszel log-rank test) in which a tumor volume greater than 1200 mm<sup>3</sup> was used as a surrogate endpoint for death. **F)** Survival analysis of mice from (C) ( $P < .001$ , two-sided Mantel-Haenszel log-rank test), in which a tumor volume greater than 1200 mm<sup>3</sup> was used as a surrogate endpoint for death. **Dotted lines**, 95% confidence intervals (E, F).

**Figure 3.** Prophylactic GCC-specific immunity against colon cancer metastases in liver. BALB/c mice were immunized with control AV or GCC<sub>ECD</sub>-AV and challenged 1 week later with  $1 \times 10^5$  CT26-GCC<sub>TM</sub> cells. Mice that were challenged with tumor cells by intrasplenic injection (day 0) were killed on day 21, and livers were collected. **A)** Livers of control- and GCC<sub>ECD</sub>-immunized mice. **B)** Liver weights of control- and GCC<sub>ECD</sub>-immunized mice. Data shown are means of  $n=5$  mice per immunization, and error bars indicate upper 95% confidence intervals ( $*P = .044$ , one-sided Student's  $t$  test). **C)** Numbers of liver nodules ( $n=11-12$  mice per immunization). Bars indicate means ( $**P = .008$ , two-sided Welch's  $t$  test).

**Figure 4.** GCC-specific immunotherapy of colon cancer metastases in lung. BALB/c mice that were immunized with control AV or GCC<sub>ECD</sub>-AV (day -7) were challenged with  $5 \times 10^5$  CT26-GCC<sub>TM</sub> cells by tail vein injection on day 0. **A)** Positron emission tomography/micro-computer tomography (PET/microCT) images of day 14 metastases with lungs outlined for clarity. **B)** PET/microCT-quantified tumor burden. Bars indicate means of  $n=5-9$  mice per immunization ( $***P < .001$ , two-sided Welch's  $t$  test). **C)** Surgically removed lungs were stained

to visualize lung nodules. **D)** Numbers of lung nodules. Bars indicate means of  $N=6-10$  mice per immunization ( $*** P < .001$ , two-sided Welch's  $t$  test). **E)** Survival analysis of mice that were challenged with tumor cells by tail vein injection following immunization as in (A-D) ( $P < .001$ , two-sided Mantel-Haenszel log-rank test). **F)** Survival analysis of therapeutic GCC<sub>ECD</sub> immunization. Mice were challenged with  $1 \times 10^5$  CT26-GCC<sub>TM</sub> cells by tail vein injection. Beginning 3 days after tumor challenge, mice were immunized sequentially with control or GCC<sub>ECD</sub> AV, RV, and VV, and survival was monitored ( $P = .024$ , two-sided Mantel-Haenszel log-rank test). **Dotted lines**, 95% confidence intervals (E, F).

**Figure 5.** Adaptive systemic cellular responses to GCC immunization. BALB/c mice were naïve or immunized with LacZ-AV or GCC<sub>ECD</sub>-AV, and sera and splenocytes were collected 10–14 days later for quantification of antibody and T cell responses. **A)** Enzyme-linked immunosorbent assay (ELISA) analysis of AV-specific IgG antibody responses in immunized BALB/c mice. Bars indicate means at reciprocal serum dilutions of 200, 400, 800, and 1600 of  $N=4$  mice per group, and error bars indicate upper 95% confidence intervals (CIs). **B)** Absence of GCC<sub>ECD</sub>-specific IgG antibody responses in immunized BALB/c mice by ELISA. Bars indicate means at reciprocal serum dilutions of 25, 50, 100, and 200 of  $N=4$  mice per group and error bars indicate upper 95% CIs. Anti-hexahistidine mouse IgG ( $\alpha$ His) served as a positive control (concentrations of 200, 100, 50, and 25 ng/mL  $\alpha$ His IgG). **C)** AV-specific CD4<sup>+</sup> T cell responses in immunized BALB/c mice measured by IFN $\gamma$  ELISpot. Data indicate pooled

analysis of N=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of two independent experiments. **D)** Absent GCC<sub>ECD</sub>-specific CD4<sup>+</sup> T cell responses in immunized BALB/c mice measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of two independent experiments. **E)**  $\beta$ -galactosidase-specific CD8<sup>+</sup> T cell responses in LacZ-AV-immunized BALB/c mice measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of four independent experiments (\*\*\*  $P < .001$ , two-way ANOVA). **F)** GCC<sub>ECD</sub>-specific CD8<sup>+</sup> T cell responses in GCC<sub>ECD</sub>-AV-immunized BALB/c mice measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of four independent experiments (\*\*\*  $P < .001$ , two-way ANOVA). Symbols indicate means in (C–F)

**Figure 6.** Differential systemic immune cell tolerance to GCC. **A)** Enzyme-linked immunosorbent assay (ELISA) analysis of AV-specific IgG antibody responses in GCC<sup>+/+</sup> (+/+) or GCC<sup>-/-</sup> (-/-) C57BL/6 mice 10–14 days after immunization with AV or a GST-fusion protein (negative control). Data represent means of n=8 AV-immunized mice per genotype or pooled samples of three control-immunized mice per genotype. Error bars indicate 95% confidence intervals (CIs). **B)** ELISA analysis of GCC<sub>ECD</sub>-specific antibody responses in +/+ or -/- C57BL/6 mice 14



days after immunization with GCC<sub>ECD</sub>-AV or LacZ-AV. Responses from individual GCC<sub>ECD</sub>-immunized mice are shown, and those from controls are presented as means (n=3 per group,  $P < .001$ , two-way ANOVA for GCC<sub>ECD</sub>-AV – /– vs all other groups). Data are representative of three independent experiments. **C)** AV-specific CD4<sup>+</sup> T cell responses in GCC<sub>ECD</sub>-AV-immunized +/+ or –/– C57BL/6 mice measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of four independent experiments. **D)** GCC<sub>ECD</sub>-specific CD4<sup>+</sup> T cell responses in LacZ-AV- or GCC<sub>ECD</sub>-AV-immunized +/+ and –/– C57BL/6 mice measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of four independent experiments ( $*** P < .001$ , two-way ANOVA for GCC<sub>ECD</sub>-AV –/– vs all other groups). **E)**  $\beta$ -galactosidase-specific CD8<sup>+</sup> T cell responses in GCC<sup>+/+</sup> C57BL/6 mice following LacZ-AV immunization and measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of six independent experiments ( $*** P < .001$ , two-way ANOVA). **F)** GCC<sub>ECD</sub>-specific CD8<sup>+</sup> T cell responses in GCC<sup>+/+</sup> C57BL/6 mice following GCC<sub>ECD</sub>-AV immunization and measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of triplicate measurements and are representative of six independent experiments ( $*** P < .001$ , two-way ANOVA). **G)**  $\beta$ -galactosidase-

specific CD8<sup>+</sup> T cell responses in +/+ and -/- C57BL/6 mice following LacZ-AV immunization measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of two independent experiments. H) GCC<sub>ECD</sub>-specific CD8<sup>+</sup> T cell responses in +/+ and -/- C57BL/6 mice following GCC<sub>ECD</sub>-AV immunization measured by IFN $\gamma$  ELISpot. Data indicate pooled analysis of n=2 mice per group, and error bars indicate 95% CIs of two independent experiments (\*  $P = .019$ , two-way ANOVA). Symbols indicate means in (A-H).

**Figure 7.** Absence of autoimmunity upon systemic GCC<sub>ECD</sub> immunization. **A)** Serum anti-nuclear antibodies (ANA) quantification from naïve, LacZ-AV-, or GCC<sub>ECD</sub>-AV-immunized C57BL/6 or naïve MRL/MpJ-Fas<sup>lpr</sup>/J mice. Data represent means and upper 95% confidence intervals. **B)** Histopathological analysis of tissues collected from BALB/c mice immunized with control or GCC<sub>ECD</sub>-AV or control or GCC<sub>ECD</sub>-ARV (sequential adeno-, rabies, and vaccinia virus from Figure 2, C-F). Images are representative sections from three mice per immunization group. Bar = 200  $\mu$ m.

**Table 1. Serum chemistries of AV-immunized GCC<sup>+/+</sup> and GCC<sup>-/-</sup> mice\***

Analyte	Control-AV		GCC <sub>ECD</sub> -AV		Normal Range	System
	GCC <sup>+/+</sup> (n=5)	GCC <sup>-/-</sup> (n=6)	GCC <sup>+/+</sup> (n=6)	GCC <sup>-/-</sup> (n=5)		
Total protein, g/dL	4.9	4.8	4.9	4.8	4.4–6.2	Liver
Glucose, mg/dL	206	209	197	190	186–265	Pancreas
Creatinine, mg/dL	0.2	0.1	0.1	0.1	0.2–0.7	Kidney
Albumin, g/dL	2.7	2.7	2.9	2.7	2.6–4.6	Liver
Na, meq/L	136	135	138	139	147–167	Electrolytes
Phosphorous, mg/dL	7	6	7	7	6–13	Kidney
Alanine transaminase, u/L						
[Jeannine: ala. trans and asp. trans below are u (units) per L not mu/L]	41	28	35	25	24–140	Liver, heart, skeletal muscle
Cholesterol, mg/dL	73	80	69	72	63–174	Liver
Total bilirubin, mg/dL	0.3	0.2	0.3	0.2	0.0–0.9	Heme catabolism, cholestasis
Aspartate transaminase, u/L	135	99	103	71	69–191	Liver, heart, skeletal muscle
Triglycerides, mg/dL	58	57	53	67	71–164	Lipids
Cl, meq/L	99	97	100	100	104–120	Electrolytes
Blood urea nitrogen, mg/dL	19	19	24	19	19–34	Kidney

\*Values indicate the mean for each group. No statistically significant differences

were seen between groups.